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Comprehensive chemical-toxicological research of copper(II) sulfate solutions containing reduced glutathione

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The composition and toxicological properties of copper(II) sulfate solutions containing reduced glutathione (GSH) have been studied. It was found that complex compounds Cu^{2+} with GSH are formed in the solution in the Cu(II):GSH molar ratios 1:1, 1:2, 1:3, 1:4. Simultaneously with the formation of complexes in solutions, oxidation-reduction reactions occur, accompanied by the formation of active forms of oxygen and oxidized glutathione (GSSG). All the described processes lead to a decrease in the active concentration of Cu^{2+} . The toxicological properties of solutions with a molar ratio of Cu(II):GSH 1:1, 1:2, 1:4 were determined. For this purpose, test objects of different systematic accessory were used: cyanobacterium *Nostoc linckia* 271, crustaceans *Daphnia magna* Straus, infusoria *Raramecium caudatum* Ehrenberg and bioassay “Ecolum”. The chemical composition of solutions was correlated with toxicity. Reduced glutathione has a protective effect on daphnia, which is most pronounced in the variant 1Cu(II):4GSH. However, the level of viability of cyanobacteria, in contrast to hydrobionts, decreases in the 1:1 → 1:2 → 1:4 range. The decrease is due to the bioaccumulation of copper, which increases with the increase in the fraction of reduced glutathione. Such an effect may be formed by complex compounds, which is consistent with the literature data. For *D. magna*, such a relationship is not observed, the resulting compounds and reduced glutathione do not affect the accumulation of metal.

Keywords: reduced glutathione, copper(II) ions, bioassay, active forms of oxygen.

Glutathione is a tripeptide consisting of amino acid residues: glutamic acid, cysteine and glycine. There are a reduced (GSH) and oxidized (GSSG) forms of glutathione. It is found in almost all living organisms. Glutathione acts as a broad-spectrum bioprotector, including protecting cells against the action of heavy metals (HM) [1, 2]. At the same time, there is information on the enhancement of the toxicity of the medium due to the fact that conjugates of HM ions (in particular Cu^{2+}) with GSH, are involved in the formation of products

of activation of molecular oxygen – active forms of oxygen (AFO), which are disastrous in high concentrations [3, 4].

According to the literature in a solution containing GSH and the Cu^{2+} ions, redox reaction may flow, accompanied by the formation of AFO [5–8] and complexation reactions [9, 10] to form complexes with certain properties [11–13]. The Cu^{2+} ions and GSH can simultaneously be present both inside the cells and in the surrounding environment of living organisms. The data available in the literature do not give a

general chemical-toxicological representation of solutions with different ratios of Cu^{2+} ions and GSH. Therefore, the goal of the study was to study the chemical-toxicological properties of model solutions CuSO_4 containing GSH with a different molar ratio Cu(II):GSH .

Objects of research

The composition of solutions obtained by mixing CuSO_4 solution ($C(\text{Cu}^{2+}) = 1.0 \text{ mg/dm}^3$) with a solution of GSH in the Cu(II):GSH molar ratios of 1:0, 1:1, 1:2, 1:4 (hereinafter variants), and their toxic effect on the culture of cyanobacteria (CB) *Nostoc linckia* 271, crustaceans *Daphnia magna* Straus, infusoria *Paramecium caudatum* Ehrenberg and the bioassay "Ecolum" (lyophilized bacterial preparation based on the bioluminescent strain *Escherichia coli* Migula) were studied.

Methods of research

Study of the chemical composition of model solutions

The active concentration of Cu^{2+} ions was determined by potentiometry on the ionometer I-160 MI with the ion-selective ELIS-131 Cu electrode [14], GSH by spectrophotometry with Ellman reagent on the UNICO-2800 spectrophotometer [15], dissolved O_2 concentration by amperometry with using the HI 9143 HANNA oximeter according to ISO 5814-84, AFO – using the chemiluminescence method using the BHL-07 biochemiluminometer [16].

Study of the response of organisms

Biotesting using cyanobacteria N. linckia 271 ($T = 1.2 \cdot 10^8 \text{ cells/cm}^3$)

After 24 hours and 7 days of exposure, the intensity of biochemiluminescence (IBCL) in CB on the BCH-07 biochemiluminometer was measured in the solutions under study [17], the viability of microorganisms was determined by direct counting of stained cells using a monocular Micros MC-10 microscope (comparison was made with the distilled water as a control) [18], the content of copper (in the form of Cu^{2+}) in the biomass of organisms – by the method of inversion voltammetry (IVA) on the analyzer of the brand Ecotest-VA [19, 20].

Bioassay using Daphnia magna Straus crustaceans

The duration of exposure of daphnia in model solutions was 24 days, during which the mortality and fecundity of organisms was determined according to FR.1.39.2007.03222.

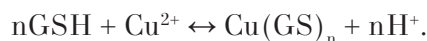
After 24 hours and 7 days, the content of copper (in the form of Cu^{2+}) in biomass was determined by the IVA method on the analyzer of the brand Ecotest-VA [19].

Bioassay using the test system "Ecolum" and infusoria Paramecium caudatum Ehrenberg

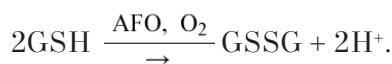
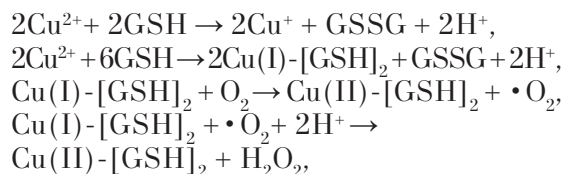
Immediately after the initial solutions of CuSO_4 and GSH were mixed in the explored ratios, the toxicity indices were determined using the Russian standard techniques of PND F T 14.1: 2: 3: 4.11-04 and PND F T 14.1: 2: 3: 4.2-98.

Results and discussion

According to the published data, during the interaction of Cu^{2+} and GSH, complexation reactions proceed according to the following scheme [9, 10]:



Redox reactions accompanied by the formation of oxidized forms of glutathione and AFO [3, 4]:



In this work, a study of the different composition of solutions of CuSO_4 with GSH was aimed at establishing the existence of complex compounds and the occurrence of oxidation-reduction reactions.

Chemical composition of CuSO_4 solutions containing GSH.

As a result of the study of model solutions of CuSO_4 containing GSH, the variants differ from each other by the presence of a specific complex compound of ratio of the reacting Cu(II):GSH components in the initial mixture: 1:1, 1:2, 1:3 and 1:4 (Fig. 1) [10, 22]. The composition of complex compounds is given from the considerations that glutathione replaces water in the coordination sphere of copper.

Data obtained by spectrophotometry showed that the compounds of Cu(II) with GSH begin to form at a 2Cu(II):1GSH molar ratio, assuming GSH can act as a bidentate ligand. As the fraction of GSH increases, the optical density of the resulting compounds increases and reaches a maximum at a ratio of 1:4. It was not possible

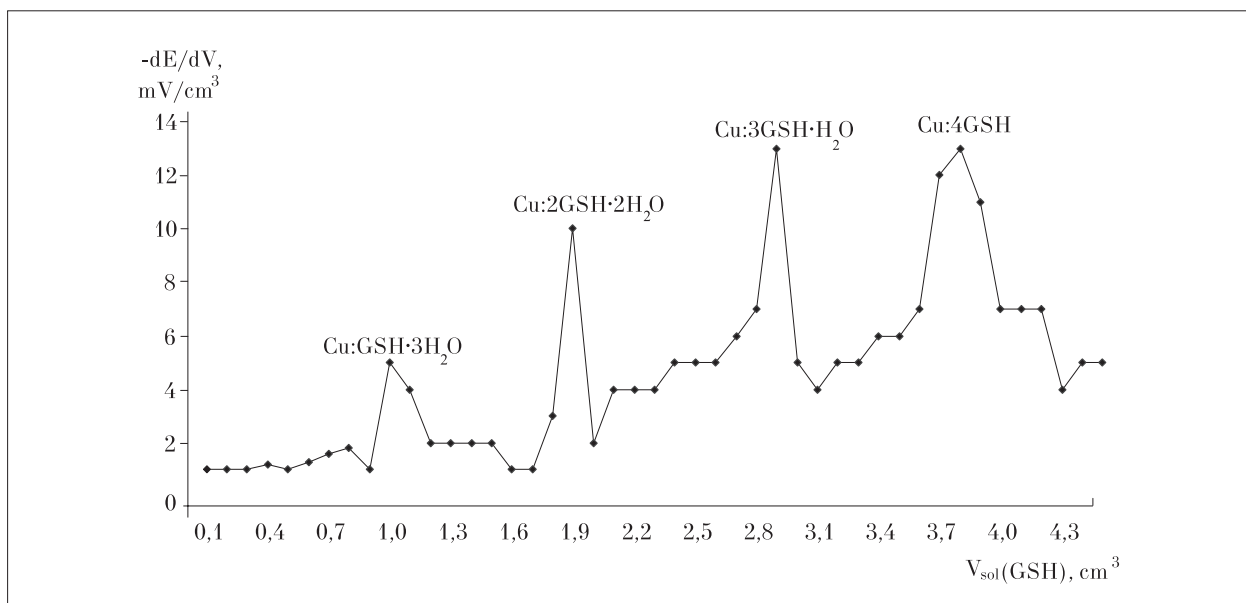


Fig. 1. Curve of potentiometric titration of $1 \cdot 10^{-5}$ M solution of CuSO_4 with $50 \cdot 10^{-4}$ M solution of GSH

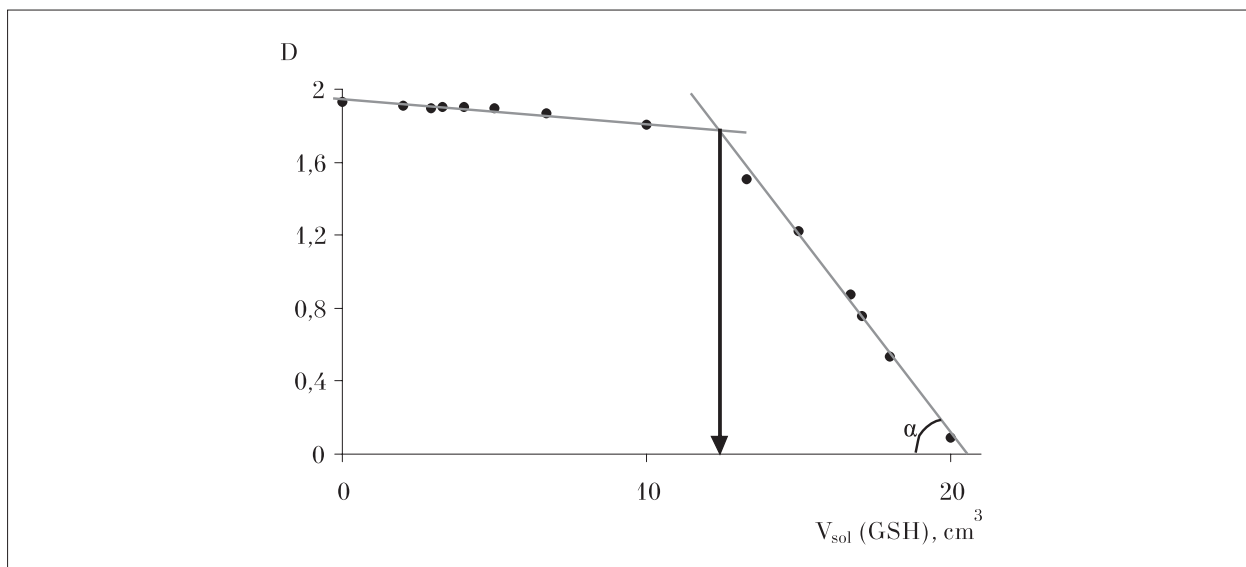


Fig. 2. Graph of isomolar series for determining the composition of the Cu(II) complex with GSH in solution. $C_{\text{GSH}} = C_{\text{Cu(II)}} = 1 \cdot 10^{-5}$ M, $\lambda = 208$ nm, and α is the slope angle of the saturation curve

to determine by this method the areas related to the different complexes of Cu^{2+} with GSH. This is due to the fact that the wavelengths at which the maximum absorption of solutions of individual compounds are observed are very close to each other, which does not allow us to reveal the composition of the complexes in an aqueous solution by this method.

The method of isomolar series showed that in the solution there is a compound corresponding to the molar ratio $1\text{Cu(II)}:1\text{GSH}$ (Fig. 2). The conclusion about the existence of a compound of this composition in the aqueous solution is

confirmed by the calculation of the slope of the saturation curve, which is numerically equal to the number of ligands ($\text{tg } \alpha = 1.19$).

Thus, the results obtained by potentiometry and spectrophotometry confirm and supplement each other. It was found that compounds Cu^{2+} with GSH with a molar ratio $\text{Cu(II)}:\text{GSH}$ 1:1, 1:2, 1:3, 1:4 are formed in the solution. To determine the composition of aqueous solutions of CuSO_4 with GSH at low concentrations, the potentiometry method is the most optimal.

The formation of copper compounds with GSH leads to a decrease in the concentra-

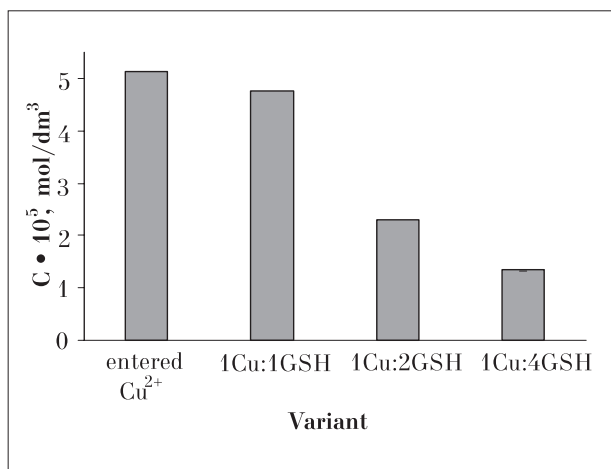


Fig. 3. Concentration of Cu²⁺ in CuSO₄ solutions containing GSH in the examined variants

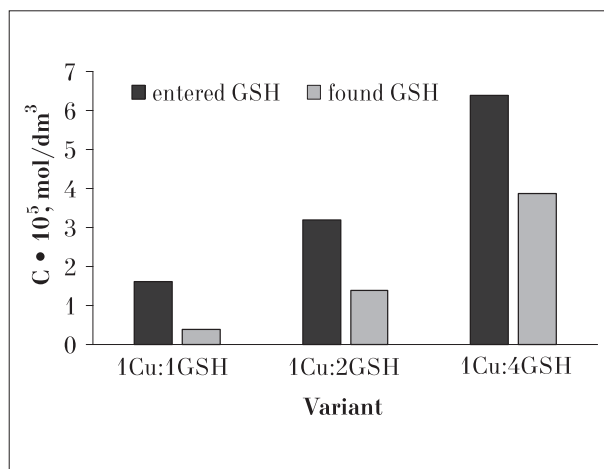


Fig. 4. Concentration of GSH in CuSO₄ solutions containing GSH in the examined variants

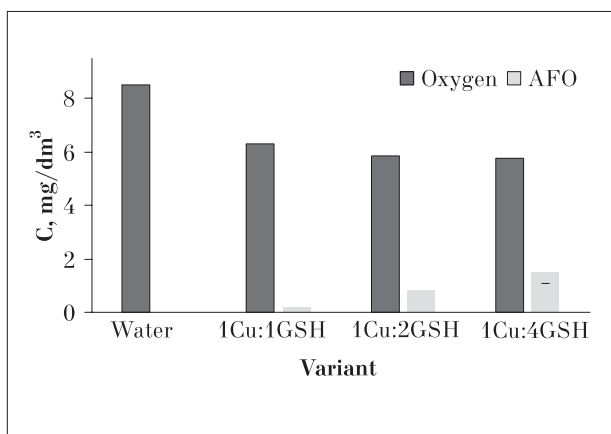


Fig. 5. Concentrations of O₂ and AFO in solutions of CuSO₄ containing GSH in the examined variants

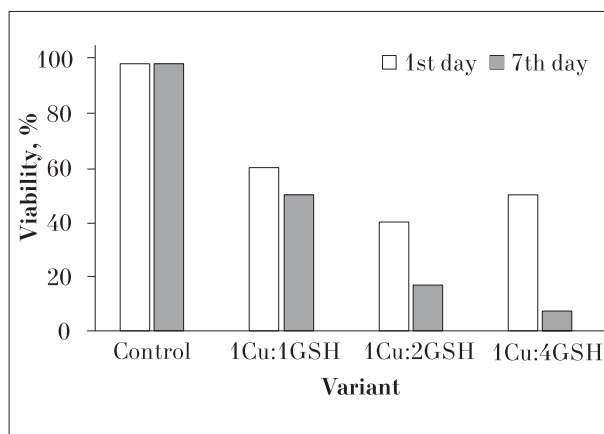


Fig. 6. Viability of *N. linckia* after contact with the solutions under study

tions of the initial components (Cu²⁺ and GSH) in the solution compared to their entered amounts (Fig. 3, 4). The value of the active concentration of Cu²⁺ decreases with the increase in the amount of GSH added. So, in the 1:1 variant, the active concentration of metal ions is 92.4±2.8% of the initial concentration, at 1:4 this index is reduced to 26.0±0.8% ($R = -0.90$, $P = 0.90$, $n = 3$) (Fig. 3). In parallel with the increase in GSH addition, the absolute value of the peptide found during the analysis increases (Fig. 4). The value characterizing the ratio of $[GSH]_{\text{found}}/[GSH]_{\text{entered}}$ in the series of options 1:1 → 1:2 → 1:4 increases from 0.25 to 0.60.

The difference in the amount of GSH found with the Ellman reagent after mixing from its entered value indicates a decrease in the concentration of Cu²⁺ and GSH not only due to the complexation processes. This statement is confirmed by the fact that in the 1:1 → 1:2 →

1:4 series, the concentration of O₂ dissolved in water significantly decreases with respect to the control (Fig. 5). The decrease is due to the consumption of O₂ for the oxidation of GSH ($R = -0.85$) and the formation of AFO, the concentration of which increases synchronously with the decrease in the O₂ concentration ($R = -0.93$). In the reactions of formation of AFO, Cu²⁺ ions can participate, as evidenced by a decrease in their concentration and an increase in the concentration of AFO ($R = -0.96$).

Effect of solution composition on toxicity

Theoretically, the bonding of copper ions Cu²⁺ to stable complexes should lead to a decrease in the toxic effect due to metal ions. However, the exposure of CB in glutathione-containing solutions of CuSO₄ within 24 hours leads to a decrease in the viability of CB in all variants compared to the control by 40–60% (Fig. 6). The toxicity level of solutions of variants 1:2 and 1:4 is interpreted as “toxicity”, since the number of

viable cells of CB after contact with these model solutions is less than $50 \pm 3\%$. By the seventh day in variants 1:2 and 1:4, the vitality is reduced to 17 ± 3 and $8 \pm 1\%$, respectively.

IBCL of the culture of *N. linckia*, in comparison with the control, decreases particularly noticeably in the 1:2 and 1:4 variant on the one day of exposure. After 7 days, the IBCL increases (Fig. 7) and exceeds the values in the control in all variants. The toxic effect in the first day was manifested in suppression, and on the seventh day in stimulation of IBCL. Stimulation of IBCL against the background of a decrease in viability can be explained by the course of oxidation-reduction processes. Essential importance belongs to the AFO, which arise as a response to the action of the stress factor [21, 22] and is capable of triggering chain reactions that last a fairly long time even after the death of the organism. The death of the organism promotes the course of reactions. This is explained by the fact that the work of many systems that attenuate

flares of redox reactions in the state of normal functioning when the organism is alive, almost completely stops after its death. In addition, AFO can be formed as a result of processes that are not related to the vital activity of the organism [8].

It was found that 7 days exposure of *N. linckia* with glutathione-containing solutions of CuSO_4 results in copper accumulation in biomass in amounts of $0.005\text{--}0.011$ mg/g of CB (in the control variant 0.00100 ± 0.00012 mg/g of CB). There is a direct relationship between the concentration of GSH in the solution and the copper content in the CB ($R = 0.97$). So in the 1:1 variant, the copper content is 0.0060 ± 0.0018 mg/g of CB, and in the variant 1:4 – 0.011 ± 0.003 mg/g of CB. With an increase in the copper content, the viability of the CB decreases ($R_{7\text{days}} = -0.80$).

Rapid methods of biotesting using *P. caudatum* and the bacterial bioassay “Ecolum” yield consistent results: a copper salt solution without glutathione and 1:1 belong to the third toxicity group (the sample is highly toxic), option 1:2 – second

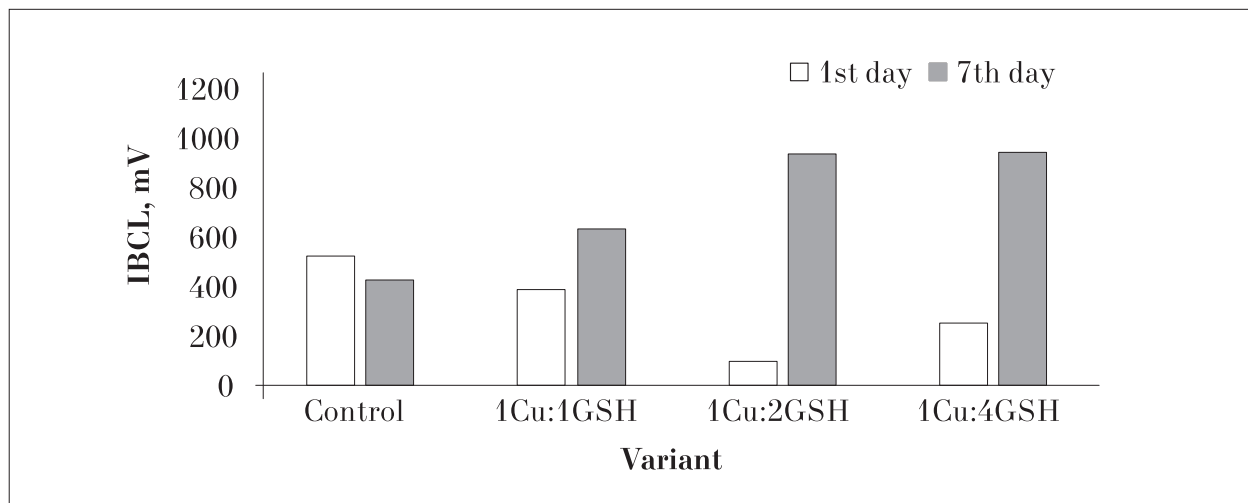


Fig. 7. IBCL of *N. linckia* after contact with the solutions under study

Table

Characteristics of the toxicity of CuSO_4 solutions containing GSH

Variant	Infuzoria <i>P. caudatum</i>		Test system “Ecolum”		Crustaceans <i>D. magna</i>		
	T index	T group	T index	T group	Duration of exposure / mortality, % of control		Fertility, % of control
					96 h	24 days	
1:0	0.78 ± 0.015	III	99.79 ± 0.03	III	100	–	–
1:1	0.85 ± 0.015	III	99.83 ± 0.09	III	100	–	0
1:2	0.66 ± 0.021	II	29.98 ± 9.17	II	53.3	70	0
1:4	0.26 ± 0.06	I	0.19 ± 4.87	I	0	3.3	101

Note: “–” – the death of daphnia occurred before the time of recording the indicator.

group (sample is medium toxic), 1:4 – first group (sample is nontoxic) (Table).

The results of establishing acute toxicity according to *D. magna* are consistent with the data obtained in the rapid bioassays (Table). Solutions 1:0, 1:1 and 1:2 had the maximum toxic effect, manifested in high mortality of the crustaceans in 96 hours of the experiment. The death time in the experiments naturally increased from several hours to the end of the acute experiment (96 hours) in the 1:0 → 1:1 → 1:2 series. In the 1:2 version, after only 96 hours, only half of the animals died. With the remaining daphnia, the experiment was continued. The crustaceans had a significant developmental retard compared to the control: a decrease in body size, pallor of the integuments, and disappearance of droplets of fat were observed. In addition, daphnia were infertile, brood chambers were not formed. As a result, in version 1:2, two main criteria for chronic toxic effects were realized: the death of individuals in 24 days significantly exceeded 20% (up to 70%), the ability to reproduce was not noted.

Reproduction of daphnia in a long-term experiment (24 days) was observed only in version 1:4. Fertility was almost equal to the control values. The copper content in the biomass of test objects on the 7th day of the experiment did not differ significantly from the control and was 0.03–0.04 mg/g of organisms.

Summarizing experiments carried out with the help of hydrobionts, it can be argued that glutathione has a protective action, which was most pronounced in the 1:4 variant.

Conclusions

As a result of studying the composition of CuSO_4 solutions containing GSH, it was found that complex compounds are formed in aqueous solutions, the composition of which corresponds to the molar ratio Cu(II): GSH to 1:1, 1:2, 1:3 and 1:4. The results of the study of the composition of solutions confirmed the literature data that in addition to the processes of complexation in glutathione-containing solutions of CuSO_4 , oxidation-reduction reactions associated with the formation of active forms of oxygen and GSSG can occur. Consequently, the toxicological properties of solutions can be manifested through the formation of complexes and products of redox reactions against the background of a decrease in the active concentration of Cu^{2+} .

2. Investigation of toxicological properties of solutions showed that for *D. magna*, *P. cau-*

datum and *E. coli*, an increase in the proportion of GSH with respect to Cu^{2+} ions leads to a decrease in toxicity, a reverse reaction is observed in *N. linckia*. The combination of chemical and toxicological methods has shown that the presence of GSH in CuSO_4 solutions influences the penetration of copper into living cells, namely *N. linckia*, but the formed compounds do not affect the accumulation of metal in *D. magna*. Thus, reduced glutathione has bioprotective properties, but the degree of their manifestation depends on the specific features of organisms.

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Some aspects of aluminum detoxifying in plants: phytotoxic and genotoxic effects

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The separate and combined impacts of aluminum, manganese and silver ions on onion (*Allium cepa* L.) have been studied. The experiments have been performed in several series with different solutions of metal salts: AlCl₃ • 6H₂O, KMnO₄, MnCl₂ • 4H₂O for the first and second series and Al(NO₃)₃ and AgNO₃ – in third series. The ion concentra-